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(54) Title: GENETICALLY MODIFIED PLANTS WITH ALTERED STARCH

(57) Abstract

Starch of wheat and maize plants is transformed by the introduction of a chimaeric gene comprising a glycogen branching enzyme coding sequence under the control of a promoter directing expression and a terminator. A transit peptide for translocation of the glycogen branching enzyme to the plant plastid may also be included in the chimaeric gene. Starch has altered processing characteristics, in particular a decreased chain length.

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Genetically Modified Plants with altered Starch

This invention relates to genetically modified plants, and in particular to genetically modified maize and wheat. The genetically modified plants have an altered starch synthesising ability following the introduction, by recombinant DNA techniques, of one or more gene sequences coding for enzymes in the starch or glycogen biosynthetic pathway into the plant.

Starch is a complex polymer of glucosyl residues. It is the major form in which carbohydrate is stored in the tissues and cells of most species of higher plants. It is accumulated in the leaves of plants during the day as a result of photosynthesis and is used to supply the needs of the plant for energy and biosynthesis during the night. Starch is also accumulated in non-photosynthetic cells, especially those involved in reproduction such as in seeds, fruits and tubers. Therefore, starch is of great importance to the productivity of the plant and its survival.

Starch is also highly significant to man. Firstly, it forms a major component of animal diets, supplying man and his domestic animals with a large portion of their carbohydrate intake. Secondly, the type of starch in a plant affects the quality of the processed plant product. Thirdly, starch is used industrially in the production of paper, textiles, plastics and adhesives, as well as providing the raw material for some bio-reactors. Starch from different species have preferred uses. On a world scale, starch producing crops are agriculturally and

economically by far the most important, and these crops include wheat, maize, rice and potatoes. The type of starch will affect the quality of a processed product and the profitability of the processed crop. In addition, the quantity and quality of starch present in the harvested organ of a plant will affect the gross yield and the processing efficiency.

In plants, i.e. vascular plants, the starch consists of linear chain and branched chain glucans known as amylose and amylopectin respectively. Starch with various amounts of amylose and amylopectin are found in different plants. Typically, plant starch contains 10-25% amylose, the remainder being amylopectin, the branched chain glucan. Amylopectin contains short chains and long chains, the short chains ranging from 5-30 glucose units and the long chains ranging from 30-100 glucose units, or more. It is thought that the ratio of amylose to amylopectin and the distribution of short to long chains in the amylopectin fraction affect the physical properties of starch, e.g. thermal stabilisation, retrogradation and viscosity. These properties also affect the utility of starch, as mentioned above. Starches from different plants have different properties, which also affects their suitability for processing under certain conditions and for certain uses. It can be seen, therefore, that modifying the starch generated in a plant can have particular utility in the downstream processing or the yield of the starch in the plant storage organ.

Waxy corn starch lacks amylose and this starch has unique properties. Also, most mutations in the waxy locus of maize, which encodes starch granule bound synthase I (GBSSI), result in

plants which produce much reduced amylose. When no functioning GBSSI is synthesised in the homozygous waxy mutant it also lacks amylose (Echt & Schwartz, 1981).

The genetic modifications of the present invention produce altered starch composition and properties, which properties are ideally beneficial in terms of starch processing.

In the last few years this concept of modifying starch properties has been postulated and put into practice in varying degrees. In the patent literature International Patent Application, Publication No. WO 94/11520 (Zeneca) described constructs having a target gene which encodes an enzyme involved in the starch or glycogen biosynthetic pathway under control of a gene switch, for example, a chemical or temperature controlled on-off mechanism. Various crops were postulated as being suitable for use in the method but no plant transformation was actually carried out. Some constructs were made but no examples or results were given. International Patent Application, Publication No. 94/09144 (Zeneca) was very similar to the just described application. Only the first steps in the transformation process were demonstrated. No results are given for any plant, and only the transformation of tomato is described with reference to the exemplary methodology, although other plants are mentioned. International Patent Application, Publication No. WO 92/11376 (Amylogene) described introducing antisense genes for GBSSI into potatoes to down regulate amylose production with the intention of producing a potato with practically no amylose-type starch. Whilst great detail is given of methodology, no actual results from transformed plants

are given and no plant transformations other than potato are postulated. Only a small number of constructs are actually produced to enable one to carry out the invention. The results for potato were eventually published in the scientific literature by Visser *et al* in 1991. Increases in the amylopectin content of the starch were seen. Further scientific papers on altering GBSSI in potato using antisense GBSSI constructs, e.g. Visser *et al* (1991) and Kuipers *et al* (1994), have shown actual transformation and alteration of starch composition.

In terms of successful transformation using non-plant derived starch-related genes, in International Patent Application, Publication No. WO 92/11382 (Calgene) and their later publication (Shewmaker *et al*, 1994) potato was actually transformed with *E. coli glgA* (Glycogen synthase) and *E. coli glgC* (ADPG pyrophosphorylase). Higher specific gravity measurements were obtained from transformed potato plants compared with two control events, as well as altered starch characteristics.

It can be seen, therefore, that work to date has involved introducing certain genes involved in glycogen biosynthesis specifically into potato. The effects and their potential usefulness for other plants and other non-plant derived starch-related genes has only been postulated.

This invention seeks to transform cereal crops and specifically wheat and maize with an enzyme involved in the synthesis of microbial glycogen, namely glycogen branching enzyme (E.C. 2.4.1.18).

This invention also seeks to identify properties of the starch in these transformed plants which are particularly useful and/or advantageous in the downstream processing of starch or the plant itself.

The present invention provides transgenic wheat or maize plants, said plants having therein a chimaeric gene comprising a promoter, a coding sequence for glycogen branching enzyme, and a terminator.

As used herein, the term chimaeric gene refers to a combination of nucleic acid sequences for each part of the chimaeric gene, which sequences have been engineered into relationship by recombinant DNA techniques, which sequences may also be in their separate parts endogenous or exogenous to the plant into which the chimaeric gene is to be introduced.

A construct and a chimaeric gene comprising nucleic acid causing the expression of the sequence above mentioned are also aspects of the invention.

Plant cells containing a chimaeric gene comprising a nucleic acid sequence encoding glycogen branching enzyme are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a chimaeric gene according to the invention.

The present invention also provides a method of altering the starch in maize or wheat plants, the method comprising the steps of stably introducing into the plant genome a nucleic acid sequence encoding glycogen branching enzyme under the direction of a suitable promoter and a suitable terminator, and regenerating a plant having an altered genome.

The present invention also provides a starch obtained from said transformed wheat or maize, said starch having an altered chain length and/or processing property compared with control starch from a non-transformed plant.

The chain length and/or branching of the starch may be increased or decreased. Evidence to date suggests that the chain length is decreased, i.e. branching probably increases. Other parameters which may be altered include the degree of retrogradation, the viscosity, the pasting temperature, the gelling temperature, each of which may be increased or decreased. The starch may also have modified properties for chemical derivitisation.

Preferably the promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant. The promoter may be heterologous or homologous to the plant. Preferably the promoter directs expression to the endosperm of the seed. A preferred promoter is the high molecular weight glutenin (HMWG) gene of wheat. Other suitable promoters will be known to the skilled man, such as the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin, for example.

Preferably the chimaeric gene also contains a sequence that encodes a transit peptide which provides for translocation of the glycogen branching enzyme and/or a marker gene or other coding sequence to the plant plastid. Suitable transit peptides include those from the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) from pea, maize or sunflower, for example. Combinations of transit peptides may

also be used. Other suitable transit peptides for transporting to the amyloplast will be known to those skilled in the art such as the transit peptide for the plant plastid acyl carrier protein (ACP) or for GBSSI.

The coding sequence encoding glycogen branching enzyme is advantageously a sequence obtained from a microorganism, such as a unicellular organism, algae, or bacteria, which sequence has the necessary ability to encode glycogen branching enzyme, or alternatively a mammalian sequence.

Suitably the glycogen branching enzyme is derived from a bacterial source such as *E. coli* (for example, Baecker, P.A. *et al*, 1983 or Kumar, A. *et al* 1986), *Agrobacterium* (Uttaro, A.D., & Ugalde, R.A. 1994), *Salmonella* (Leung, P.S.C. & Preiss, J. 1987), or *Bacillus* (Kiel, J.A. *et al 1994)*. Standard methods of cloning by hybridisation or polymerase chain reaction (PCR) techniques may be used to isolate the sequences from such organisms: for example, molecular cloning techniques such as those described by Sambrook, J. *et al* 1989 and the PCR techniques described by Innis, M.A., *et al* 1990. Other microbial sequences may be obtained in a similar manner.

The chimaeric gene may comprise one or more additional coding sequences from the starch or glycogen biosynthetic pathway, such as, for example, glycogen synthase (EC 2.4.1.21).

The transformation technique for the method of the invention are advantageously direct DNA transfer techniques, such as electroporation, microinjection or DNA bombardment (the biolistic approach). Alternatively, plant cell transformation using plant vectors introduced into plant pathogenic bacteria,

such as *Agrobacterium*-mediated transfer (Cheng, M. *et al* (1997)), may be used. In both methods selectable markers may be used, at least initially, in order to determine whether transformation has actually occurred. Useful selectable markers include enzymes which confer resistance to an antibiotic, such as gentamycin, hygromycin, kanamycin and the like. Alternatively, markers which provide a compound identifiable by a colour change, such as GUS, or luminescence, such as luciferase, may be used.

The chimaeric gene may also comprise a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed. The gene switch may be a chemically induced promoter or a temperature controlled promoter.

In order that the invention may be easily understood and readily carried into effect, reference will now be had, by way of example, to the following diagrammatic drawings in which:

Figure 1 shows a map of the plasmid pJIT117 used in the preparation of the plasmid of Figure 2;

Figure 2 shows a map of the plasmid pBS17R used in the sticky feet polymerase chain reaction;

Figure 3 shows a diagrammatic representation of the steps in the sticky-feet polymerase chain reaction;

Figure 4 shows a map of the plasmid pBSHMWGP used in the preparation of the plasmid of Figure 6;

Figure 5 shows a map of the plasmid pDV02000 used in the preparation of the plasmid of Figure 6;

Figure 6 shows a map of the plasmid pDV03000 used in the

preparation of the plasmid of Figure 7;

Figure 7 shows a map of the plasmid pDV03201 according to one aspect of the invention and used in the transformation process of the invention.

Figure 8 shows a standard chromatogram of glucose at 1mM concentration;

Figure 9 shows a standard chromatogram of maltose at 1mM concentration;

Figure 10 shows a standard chromatogram of maltotriose at 1mM concentration;

Figure 11 shows a standard chromatogram of maltohexaose at 1mM concentration;

Figure 12 shows a standard chromatogram of a mixture of maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose each at 1mM concentration;

The invention will now be described, by way of example, with reference to an embodiment for incorporating *glgB* from *E. coli* strain LCB618 into wheat and maize.

Example 1

Construction of *glgB* and plasmids used for particle bombardment of wheat and maize embryos.

Isolation of *E. coli* chromosomal DNA

The coding sequences for *glgB* was originally isolated by PCR using chromosomal DNA from the *E. coli* strain LCB618 as

template. *E. coli* LCB618 was obtained from the *E. coli* Genetic Stock Center, Yale University, U.S.A.

E. coli LCB618 was grown up in 100ml LB o/n at 37^{0}C. Cells were pelleted and resuspended in 9.5ml 10mM Tris-HCl, 1mM EDTA (TE) pH8.0 and 0.5ml 10% (w/v) Sodium dodecyl sulphate(SDS) and 50μl proteinase K 20mg/ml were added. The mixture was incubated at 37^{0}C for 1h to lyse cells. 1.8ml of 5M NaCl followed by 1.5ml of CTAB (cetyl trimethyl ammonium bromide)/NaCl solution (10%w/v CTAB in 0.7M NaCl) were added and the mixture incubated at 65^{0}C for 20 minutes. The lysate was extracted with an equal volume of chloroform and centrifuged at 6000g to separate the layers. The upper layer was removed to a fresh tube and DNA was precipitated by the addition of 0.6 volumes isopropanol. The DNA was removed from the solution with a sealed pasteur pipette, placed into a fresh tube and washed with 70% ethanol. The DNA was dried *in vacuo* and resuspended in TE pH8.0. The DNA was purified on a CsCl gradient.

Sticky-feet PCR

In order for the *E. coli* glycogen branching enzyme to function in plants the protein has to be transported into the amyloplast. This transport can be facilitated by attachment of a plastid transit peptide to the amino terminus of the *E. coli* polypeptide.

The coding sequence for the transit peptide (TP) from the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) pea has been cloned and the TP shown to target β-glucuronidase (GUS) protein to chloroplasts (Guerineau et al,

1988).

The plasmid pJIT117 (Guerineau et al, 1988), the map of which is shown in Figure 1, has several restriction sites downstream of the ssuTP which can be used for subcloning of coding sequences, however the subcloning must create a translational fusion between the transit peptide and the coding sequence and the Cys-Met amino acid sequence at the junction must be maintained.

We have previously used pJIT117 to attach the ssu transit peptide to the coding sequence for *E. coli* ADPG PPase *glgC16* using restriction digestion and PCR. The TP-*glgC16* DNA, herein known as SEQ. ID. No. 1, was subsequently transferred to the vector pBluescript (Stratagene Ltd., Cambridge, U.K.) to create pBS17R (the map for which is shown in Figure 2) and this plasmid was useful in generating a similar construct for *glgB*.

The *glgB* coding sequence has no convenient restriction sites at the 5' end. Therefore, to ensure that the open reading frame was in a translational fusion with the ssu transit peptide and to maintain the integrity of the Cys-Met cleavage site, plasmid pBS17R was used to substitute the *glgB* sequence for the *glgC16* sequence with a technique called sticky-feet PCR (Clackson and Winter, 1989).

This technique is explained diagrammatically with reference to Figure 3. In this technique, PCR primers are designed to the 5' and 3' ends of the acceptor sequence of chromosomal or genomic DNA and the sequences which are to be attached to the acceptor from a donator plasmid. In Step A, PCR is used to amplify the sequences which are to be inserted in the donator.

In Step B, the amplified acceptor DNA fragment is annealed to the donator plasmid which has been made single-stranded and carries uracil residues instead of thymidine residues by using a specific type of *E. coli* host. In Step C, a new strand is synthesised using the donator plasmid as template and the acceptor fragment as primer with a combination of *Taq* polymerase, T7 DNA polymerase (Sequenase) and T4 DNA ligase. The new double-stranded plasmid is a hybrid with one strand of the uracil-containing donator and one strand incorporating the acceptor fragment.

This hybrid plasmid is then transferred into a normal *E. coli* host where the uracil-containing strand is degraded and the acceptor strand replicated. A double-stranded plasmid incorporating the acceptor DNA can then be recovered. As an alternative, in Step D (not shown), the hybrid plasmid can be used in a PCR reaction with primers which will amplify out the acceptor DNA with the required fragments from the donator attached.

In this particular example, *glgB* sticky-feet primers were designed as follows:

SEQ. ID. No. 3 *GLGB*SF5 (P1)

TGGTGGAAGAGTAAAGTGCATGTCCGATCGTATCGATAGAGACGT

ssu TP 3' end *glgB* 5' end

SEQ. ID. No. 4 *GLGB*SF3 (P2)

TCGCTCCTGTTTATGCCCTAGATCTTCATTCTGCCTCCCGAACCAGCCAGA

glgC 3' end *glgB* 3' end

The PCR primers are designed to the 5' and 3' ends of the *glgB* cDNA sequence.

The 5' end primer (Seq. ID. No: 3) also has sequences which are homologous to the ssu-TP.

The 3' end primer (Seq. ID. No: 4) also incorporates sequences which are homologous to the 3' end of the *glgC* coding sequence. These primers are used in a PCR process to amplify a *glgB* fragment with extensions which will overlap onto the sequences in pBS17R. This is represented by Step A of Figure 3.

Plasmid pBS17R is made into a template for sticky-feet PCR by transferring the plasmid into the *E coli* host CJ236 (Raleigh et al., 1989). This host is deficient in the enzyme dUTPase, (i.e. dut$^-$) which results in deoxyuridine being incorporated into the DNA instead of thymidine. The absence of another enzyme uracyl N-glycosylase (ung$^-$) means that the deoxyuridines can not then be removed from the DNA.

In Step B of Figure 3, the extended *glgB* DNA (2) is annealed to the uracil-containing template which has been isolated as single-stranded DNA (3), and a new strand is synthesised as per Step C above. The new double-stranded plasmid is a hybrid (5) with one strand of the uracil-containing template (3) and the other strand consisting of the plasmid backbone and the *glgB* fragment now with ssu-TP and a 3' *glgC* fragment attached at 5' and 3' ends respectively (4).

In Step D (not shown), the hybrid plasmid is used in a PCR reaction with primers SEQ. ID. No. 5 (P3) and SEQ. ID. No. 4 (P2) which will amplify out the extended *glgB*.

With reference to Figure 3, the experimental details are as follows:

The primers *GLGBSF5* (P1) (SEQ. ID. No. 3) vs *GLGBSF3* (P2)(SEQ. ID. No. 4) were kinased and used to amplify the *glgB* open reading frame with extension sequences using *E. coli* LCB618 genomic DNA (1) as template. The DNA (2) was purified with GeneClean (BIO 101, Ltd).

The sticky-feet template DNA, single-stranded uracil pBS17R DNA (3), was isolated from 5ml overnight cultures of the *dut⁻ ung⁻ E. coli* strain CJ236.

The sticky-feet PCR reaction was carried out in 10μl volume containing 20ng ss uracil pBS17R (3); 200ng *glgB* DNA(2), 1μl x10 Taq polymerase buffer, 1.0μl 2mM mixture of dATP, dTTP, dCTP, dGTP (2mM dNTPs); 2.5 units Taq polymerase. The mix was overlayed with 30μl mineral oil and cycled once at 94^0C, 3min; 72^0C, 2 min; 40^0C, 2 min. and then cooled to room temperature. 10μl of a solution containing 2.0μl x5 Sequenase buffer (200mM Tris-HCl pH 7.5,100mM $MgCl_2$, 250mM NaCl), 1.5μl 0.1mM Dithiothreitol (DTT); 2.0μl 10mM Adenosine 5' triphosphate (ATP); 4 units T4 DNA ligase; 6.5 units Sequenase was then added and the reaction incubated at room temperature for 30 minutes.

Generation of TP-*glgB* DNA

1.0μl of the reaction containing the hybrid plasmid (3 + 4) was taken and diluted to 10μl with 10mM TE pH 8.0. 1.0μl of the diluted sample was used in a PCR reaction in order to obtain the TP-*glgB* coding sequence (Step C of Figure 3). Primers used were TPSSU5 (P3)(SEQ. ID. No. 5) vs *GLGBSF3* (P2) (SEQ. ID. No. 4).

SEQ. ID. No. 5 TPSSU5 (P3)

ACGTAGATCTATGGCTTCTATGATATCCTCTTC

The primers both have restriction sites for *Bgl*II, therefore after purification, the amplified DNA was digested with *Bgl*II and subcloned into the *BamH*I site of pDV03000 (see below).

Construction of pDV03000 vector

Transgenic wheat and maize plants are generated by particle bombardment of embryos and it is not necessary to use binary vectors. For expression of the *glgB* protein the coding sequence has to be placed under the control of an endosperm-specific promoter. One such suitable promoter is that from the High molecular weight glutenin (HMWG) gene of wheat (Bartels and Thompson, 1986). Primers *(P4)* and *(P5)* (SEQ. ID. Nos. 6 and 7 respectively) were designed so that the 430bp HMWG promoter (the nucleotide sequence of which is given in SEQ. ID. No. 2) could be isolated by PCR and subcloned via *EcoR*I and *Cla*I restriction sites into pBluescript to generate the plasmid pBSHMWGP (Figure 4).

A second set of PCR primers were designed to obtain the nopaline synthase terminator from plasmid pDV02000, the map of which is shown in Figure 5. This plasmid was previously constructed in our laboratory as an intermediate vector for the subcloning of coding sequences. The 5' primer, NTPRIME5 *(P6)* (SEQ. ID. No. 8), has a *BamH*I restriction site, while the 3'

primer, NTP3NXS2*(P7)* (SEQ. ID. No. 9), has restriction sites for *Not*I, *XhoI* and *Sac*II. The amplified DNA was digested with *BamHI* and *Sac*II and ligated into the pBSHMWGP plasmid to generate pDV03000, the map of which is shown in Figure 6.

SEQ. ID. No. 6 HMWGPRO5 *(P4)*

GACATCGATCCCAGCTTTGAGTGGCCGTAGATTTGC

SEQ. ID. No. 7 HMWGPRO3 *(P5)*

GACGAATTCGGATCTCTAGTTTGTGGTGCTCGGTGTTGT

SEQ. ID. No. 8 NTPRIME5 *(P6)*

CAGGATCCGAATTTCACCCGATCGTTCAAACA

SEQ. ID. No. 9 NTP3NXS2 *(P7)*

GACCCGCGGCTCGAGGCGGCCGCCCGATCTAGTAACATAGATGACACCGC

pDV03000 vector has the HMWG promoter-nos terminator seqeunces separated by unique restriction sites for *Eco*RI, *Pst*I, *Sma*I and *BamHI*.

Construction of pDV03201

TP-*glgB* DNA amplified from the sticky-feet PCR sample with primers TPSSU5 vs *GLGB*SF3 (Step D, Figure 3) was digested with *Bgl*II, purified and ligated into the *BamHI* site of pDV03000. Plasmid pDV03201 (the map of which is shown in Figure 7) was confirmed by restriction enzyme digestion and by sequencing of the junctions between promoter and coding sequence. *E. coli* XL1

Blue (Stratagene Ltd., U.K.) harbouring pDV03201 was deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge Science Park, Cambridge CB4 0WA under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collection of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland, GB on 14th October 1998 under accession number NCIMB 40982. The micro-organism is *E.coli* XL1 Blue: strain LCB618 containing pDV03201. The DNA for *E. coli glgB* was inserted as described aboved into pBluescript with the ssu transit peptide, the HMWG promoter and nos terminator. The vector is useful for altering starch properties.

Maize plants transformed with *glgB* recombinant gene

In the transformation step, immature maize embryos are to particle bombardment with gold particles coated with plasmid DNA, in this case pDV03201. Methods for the transformation of maize are well known in art, for example see Gordon-Kamm et al (1990) and Fromm et al (1990).

Two plasmids are used per bombardment, one plasmid carries the construct of interest, in this case pDV03201. The second plasmid carries the selectable marker which expresses the gene responsible for resistance to the herbicide Basta. Plants resistant to Basta are generally found to also have the recombinant gene of interest present.

Bombarded calli are grown on Basta selection media and surviving calli are transferred to regeneration medium. Rooted plants are transferred to soil and grown to maturity in a growth

room.

After rooted primary transformant plants (T_0) are transferred to soil and grown to maturity, maize plants are backcrosssed to produce transgenic seed which can be extracted and analysed according to Example 2. Further backcrossing is performed to introgress the transgene into elite varieties and selfing of transgenic plants is performed to obtain plants and seed which are homozygous for the transgene. Seed from these generations can also be extracted and analysed according to Example 2.

Seed from a number of backcrossed primary transformants were shown to be expressing the *glgB* protein.

Example 2

Biochemical Analysis of *glgB* transformed wheat and maize

1. Expression of *glgB* protein.

Soluble protein samples were prepared from individual wheat or maize grain derived from transformed plants. Each grain was pulverised in a pestle & mortar until a fine powder was obtained. A portion of this powder (100-200mg) was placed in an Eppendorf tube and 500μl of ice cold extraction buffer (50mM HEPES, pH 8.0; 10mM DTT; 10mM EDTA) added. The powder was homogenised with a micropestle to release soluble proteins.

The extract was centrifuged at 13000 rpm for 1 minute and the supernatant decanted into a fresh Eppendorf tube and stored on ice. The total protein content in the soluble protein sample was assayed using the Bradford dye binding method (Bradford, M.,

1976).

An aliquot of the soluble protein sample, containing 100mg total protein was placed into an Eppendorf tube and excess acetone (*ca* 1.5ml) was added to precipitate the proteins. The proteins were collected by centrifuging the sample at 13000 rpm for 5 minutes. The acetone was decanted off and the samples were air-dried until all the residual acetone had evaporated.

SDS PAGE loading buffer (4% (w/v) SDS; 12% (w/v) glycerol; 50 mM Tris-HCl pH 6.8; 2% (v/v) β-mercaptoethanol; 0.01% Serva blue G) 100μl, was added to the protein sample contained in the Eppendorf tube. Samples were boiled for 1 minute before loading onto a polyacrylamide gel.

Electrophoresis was carried out according to the method of Schagger and Von Jagow (1987). The resolving gel composition was 10% acrylamide, 3% bis-acrylamide. Gels were run at 50 V constant for 16 hours.

Separated proteins were transferred from the acrylamide gel onto PVDF membrane by electroblotting (Transfer buffer: 20% methanol; 25 mM Tris-HCl pH 8.3; 190 mM glycine. Run in a Biorad blotting apparatus at 50 V for 3 hours).

To detect expression of *glgB* the membrane was challenged with a rabbit anti-*glgB* antiserum (raised to the *glgB*-GST fusion protein purified from *E. coli*). Specific cross-reacting proteins were detected using an anti-rabbit IgG-alkaline phosphatase conjugate secondary antibody and visualised by the NBT/BCIP reaction.

NuPAGE™ Electrophoresis.

Alternatively, an aliquot of the soluble protein sample,

containing 100mg total protein was placed into an Eppendorf tube and excess acetone (*ca* 1.5 ml) was added to precipitate the proteins. The proteins were collected by centrifuging the sample at 13000 rpm for 5 minutes. The acetone was decanted off and the samples were air-dried until all the residual acetone had evaporated.

NuPAGE™ loading buffer (2% (w/v) SDS; 10% (w/v) sucrose; 25 mM Tris-HCl pH 8.5; 1% (v/v) β-mercaptoethanol; 0.5 mM EDTA; 0.02% Serva blue G250; 0.006% Phenol Red) 100 ml, was added to the protein sample contained in the Eppendorf tube. Samples were heated at 100 °C for 1 minute before loading onto a polyacrylamide gel. Electrophoresis was carried out on NuPAGE™ precast gels according to the manufacturers instructions (Novex, San Diego CA). Gels were run at 200 V constant for 60 minutes using MES SDS running buffer (20 mM MES/20 mM Tris-HCl pH 7.3; 1% (w/v) SDS; 1 mM EDTA).

Separated proteins were transferred from the acrylamide gel onto PVDF membrane by electroblotting (Transfer buffer: 20% methanol; 25 mM Bis-Tris/25 mM Bicine pH 8.3; 1 mM EDTA. Run in a Novex electroblotting apparatus at 25 V for 1.5 hours).

To detect expression of *glgB* the membrane was challenged with an rabbit anti-*glgB* antiserum (raised against *glgB*-GST fusion protein purified from *E. coli*). Specific cross-reacting proteins were detected using an anti-rabbit IgG-horse Radish Peroxidase conjugate secondary antibody and visualised using enhanced chemiluminesence (ECL) as supplied by Amersham International.

Several transformed lines were found to express a 84kDa

protein in their grain, which was not present in control grain derived from non-transformed wheat or maize plants.

2. Preparation of maize starch.

Starch was extracted from grain of separate field grown samples of two of the *glgB* expressing lines and a control line. Maize grains of each sample (3-4g) were placed in a mortar, 30ml of 1% Sodium bisulphite was added and placed on ice for 30 minutes. The grains were then gently pulverised using a pestle. The solution was filtered through a nylon filter sieve and collected in a centrifuge tube. The pulverised maize grains were re-extracted with a further 30ml of 1% Sodium bisulphite and the filtrates were combined. The filtrate was centrifuged at 6000 rpm for 5 minutes. After decanting off the supernatant, the pellet of extracted starch was resuspended in water and centrifuged at 6000 rpm for 5 minutes. This was repeated once. The resulting starch pellet was resuspended in acetone, centrifuged at 6000 rpm for 5 minutes and the supernatant decanted away. This was repeated once and the starch left to air dry. Once dried the starch was stored at -20° C.

3. Branch chain length analysis of maize starch.

Portions of the starch samples were digested with isoamylase and the resulting unbranched linear glucan chains were analysed by HPLC.

75mg of isolated maize starch was placed in a 15ml Pyrex boiling tube and suspended in 3.0 ml of water. The sample was placed in a boiling water bath for 6 minutes, occasionally

removed and vortex mixed. The sample was cooled to room temperature and 250μl of 200mM Sodium acetate, pH 3.5 and 180 units of isoamylase enzyme added. The samples were made up to a final volume of 3.8 ml with water. After mixing the sample was placed in a 37^{0}C water bath for 4 hours. The samples were occasionally vortex mixed throughout this incubation period. At the end of the incubation the sample was placed in a boiling water bath for 2 minutes, and then allowed to cool to 4^{0}C. The sample was centrifuged at 3,400 rpm for 20 min. The resulting supernatant was transferred to Eppendorf tubes and centrifuged at 13000 rpm for 15min. and finally the sample was filtered through a 0.2mm syringe filter, and stored at 4^{0}C until required.

Separate isoamylase digest samples were normalised to a constant total glucan content by digesting a portion of the sample to glucose using α-amylase and amyloglucosidase.

Two 100μl aliquots of isoamylase digested starch were placed in two separate Eppendorf tubes (one is to be used as a blank). To one aliquot was added: 500μl of 200mM Sodium acetate pH 4.8; 50μl of α-amylase solution containing 10 units of α-amylase; 100μl of amyloglucosidase solution containing 10 units of amyloglucosidase and water to a final volume of 1.0 ml. To the second (blank) aliquot was added: 500μl of 200mM Sodium acetate pH 4.8 and 400μl of water. The samples were left to digest at 25^{0}C for 16 hours.

The glucose content of the digest and blanks was assayed spectrophotometrically using a coupled enzyme assay. An aliquot of the total glucose digest or the blank was added to a cuvette

containing in a final volume of 990μl 100mM HEPES, pH 8.0; 5mM $MgCl_2$; 4mM NAD; 1mM ATP and 1 unit of hexokinase. The optical density (OD) of the reaction mixture at 340 nm was measured prior to the addition of 10μl containing 1 unit of glucose-6-phosphate dehydrogenase. The OD at 340 nm was monitored until there was no further change and the difference in OD after the addition of glucose-6-phosphate dehydrogenase compared to before the addition of glucose-6-phosphate dehydrogenase was determined. This figure was used to determine the total glucose amounts in the original isoamylase digests. These samples were diluted with water to a standard concentration of 8mM total glucose and stored at 4^0C until required for HPLC analysis.

The samples were then analysed by Dionex HPLC using a Dionex PA 100 column and PED - Integrated Amperometric detection. The solvent flow rate was 1.0 ml/min and a gradient system was developed. Solvent 1 consisted of 100mM NaOH and Solvent 2 was 100mM NaOH, 0.60M Sodium acetate. The gradient profile was as shown in Table 1, with the pulsed electrochemical detection (PED) parameters shown in Tables 2.1 and 2.2.

Table 1.

Gradient profile

Event Start Time (min)	Solvent 1 (%)	Solvent 2 (%)
0	100	0
1	100	0
2	100	0
30	0	100
30.1	100	0
35	100	0

Table 2.1

Waveform table

Time (sec)	Potential (V)
0	0.1
0.5	0.1
0.51	0.6
0.59	0.6
0.6	-0.6
0.65	-0.6

Table 2.2

Integration

Begin (sec)	End (sec)
0.3	0.5

Three isoamylase digestions were performed for each sample and three aliquots of each isoamylase digest were analysed by the HPLC system. Separate chromatogram peaks were assigned to specific linear glucan sizes by reference to standard mixtures

containing linear glucans of known numbers of glucose molecules (see Figures 8-12). Peak areas were abstracted from the primary data and averaged for the replicate chromatograms.

Figures 8 to 12 are HPLC traces of standards for various sugars. The standards in Figures 8-12 allow the peak area for each peak of the inventive sample and its control to be converted to a quantitative representation of the number of glucan chains in each peak, and the position (on the x-axis) of each peak to the number of glucose residues in each chain, i.e. the chain length.. Evidence to date suggests that there is an increased number of shorter chain lengths of dp 5-8. The starch is therefore altered, which alteration affects its processing capabilities.

Example 3

Transformation of wheat

Methods for the transformation of wheat by particle bombardment are well known in the art, for example see Vasil *et al*, 1992.

Immature embryos of wheat are used to initiate embryogenic callus. The callus is subcultured and used for particle bombardment with gold particles coated with plasmid DNA.

Two plasmids are used per bombardment, one plasmid carries the construct of interest, in this case pDV03201. The second plasmid carries the selectable marker which expresses the gene responsible for resistance to the herbicide Basta. Plants resistant to Basta are generally found to also have the recombinant gene of interest present.

Bombarded calli are grown on Basta selection media and surviving calli are transferred to regeneration medium. Rooted plants are transferred to soil and grown to maturity in a growth room.

Primary transformant wheat plants (T_0) are selfed to produce transgenic seed.

Seed are extracted for protein and the protein analysed by western blotting for the presence of *E. coli glgB* polypeptide.

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Materials Abbreviations

LB - Luria broth

TF - Tris-HCl, 1mM EDTA

SDS - sodium dodecyl sulphate

CTAB - cetyl trimethyl ammonium bromide

dATP - 2' - deoxy adenosine 5' triphosphate

dTTP - 2' - deoxy thymidine 5' triphosphate

dCTP - 2' - deoxy cytosine 5' triphosphate

dGTP - 2' - deoxy guanosine

DTT - dithiothreitol

ATP - adenosine 5' triphosphate

HEPES N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

NBT - nitroblue tetrazolium

BCIP - 5-bromo-4-chloro-3-indolyl phosphate

GST - glutathion S transferase

NAD - nicotinamide adenine dinucleotide

IgG - immunoglobulin G

CLAIMS

1. A method of altering the starch in maize or wheat plants, the method comprising the steps of stably introducing into the plant genome a chimaeric gene comprising a nucleic acid sequence encoding glycogen branching enzyme under the direction of a suitable promoter and a suitable terminator, said glycogen branching enzyme being from a microorganism, and regenerating a plant having an altered genome.
2. A method according to Claim 1, wherein said nucleic acid sequence encoding glycogen branching enzyme is a sequence obtained from a unicellular organism, an alga or bacterium, which sequence has the necessary ability to encode glycogen branching enzyme.
3. A method according to Claim 1 or 2, wherein said glycogen branching enzyme is derived from *E.coli*, *Agrobacterium*, *Salmonella* or *Bacillus*.
4. A method according to Claim 1, 2 or 3, wherein said promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant.
5. A method according to any one of Claims 1 to 4, wherein said promoter is heterologous or homologous with respect to said plant.
6. A method according to Claims 1, 2, 3, 4 or 5, wherein said promoter directs expression to the endosperm of the seed.
7. A method according to Claim 6, wherein said promoter is the high molecular weight glutenin (HMWG) gene of wheat.

8. A method according to Claim 4, wherein said promoter is one or more of the group consisting of the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin.

9. A method according to any one of Claims 1 to 8, wherein said chimaeric gene also contains a sequence that encodes a transit peptide which provides for translocation of the glycogen branching enzyme and/or a marker gene or other coding sequence to the plant plastid.

10. A method according to Claim 9, wherein said transit peptide is one or more of the group consisting of the small subunit of the ribulose bisphosphate carboxylase enzyme (ssu of Rubisco) from pea, maize or sunflower, the transit peptide for the plant plastid acyl carrier protein (ACP) or the transit peptide for GBSSI.

11. A method according to any one of the preceding claims, wherein said chimaeric gene comprises one or more additional coding sequences from the starch or glycogen biosynthetic pathway.

12. A method according to Claim 11, wherein said additional coding sequence is the sequence glycogen synthase (EC 2.4.1.21).

13. A method according to any one of the preceding claims, wherein said chimaeric gene also comprises a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed.

14. A method according to Claim 13, wherein said gene switch is a chemically induced promoter or a temperature controlled

promoter.

15. Starch obtained from wheat or maize transformed according to Claims 1-14, said starch having an altered chain length and/or processing property compared with control starch from a non-transformed plant.

16. Starch according to Claim 15, wherein said chain length is decreased.

17. Starch according to Claim 15, wherein the viscosity is increased, said altered viscosity affecting the processing properties of said starch.

18. Starch according to Claim 15, wherein the degree of retrogradation of said starch is lower, said altered degree of retrogradation affecting the processing properties of said starch.

19. Starch according to Claim 15, wherein the freeze-thaw stability of said starch is improved.

20. Maize or wheat plant cells containing a chimaeric gene comprising a promoter, a coding sequence for glycogen branching enzyme, and a terminator.

21. Seed of a maize or wheat plant transformed in accordance with any one of Claims 1-14.

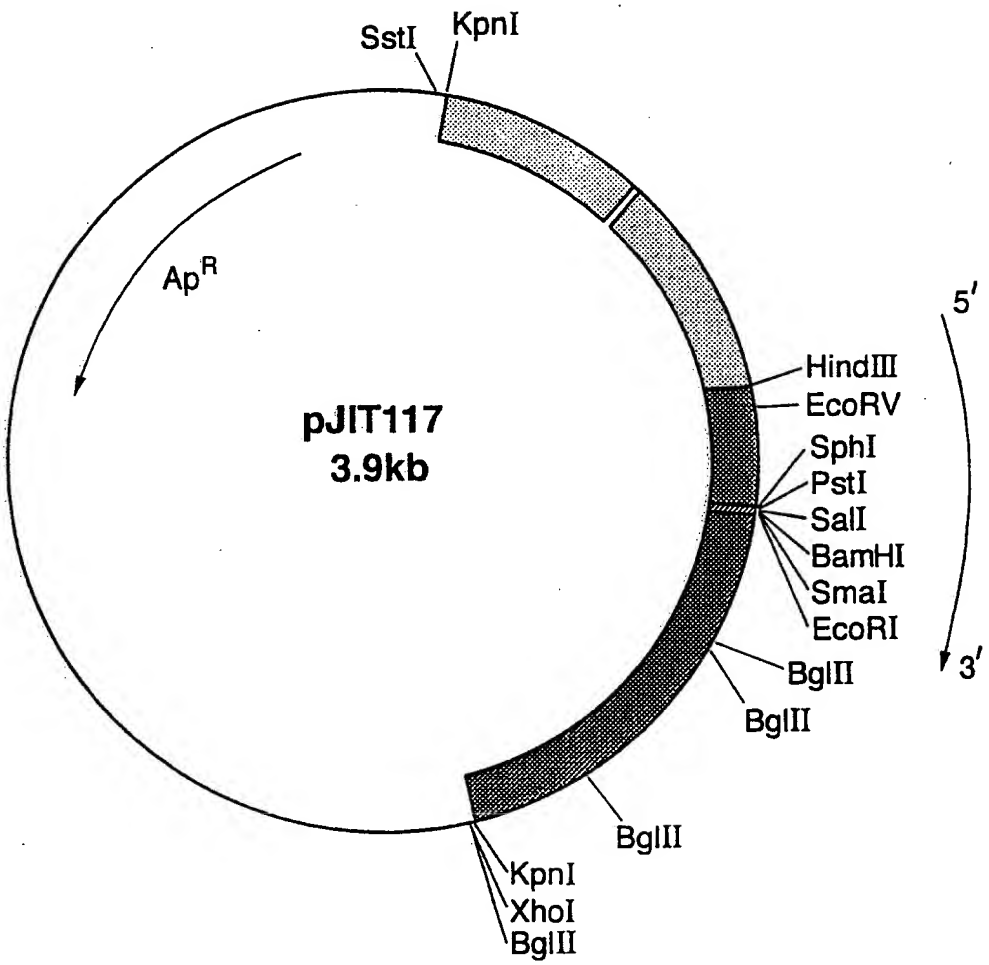
22. Maize or wheat plants or cells transformed according to any one of Claims 1-14 and containing starch having a decreased chain length.

23. A construct as described in Figure 7 and deposited under NCIMB Accession No. 40982.

24. A construct comprising a promoter-gene fragment-terminator cassette comprising a transit peptide and coding sequence

for glycogen branching enzyme.

Fig.1.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Polylinker
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pUC based plasmid

Fig.2.

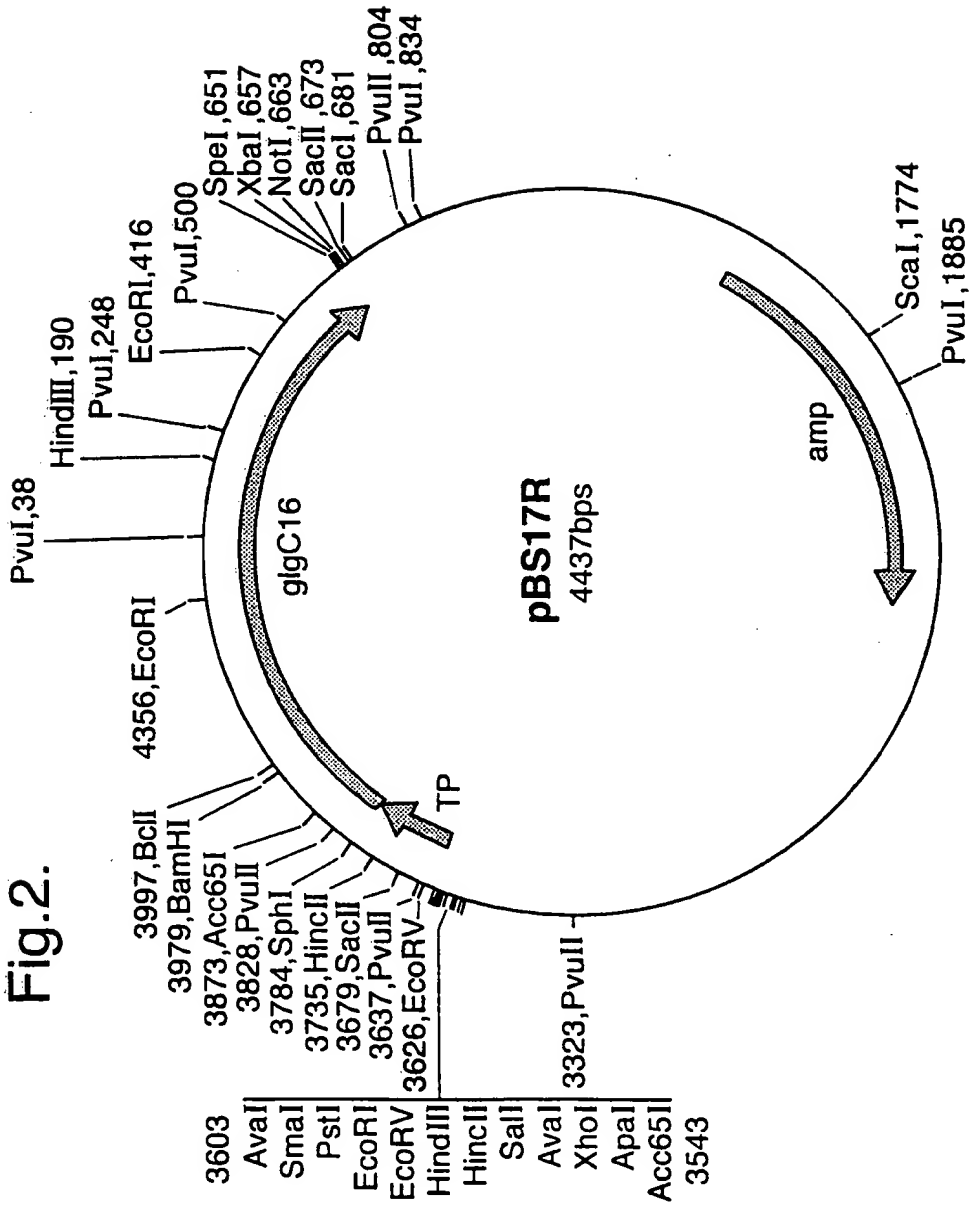
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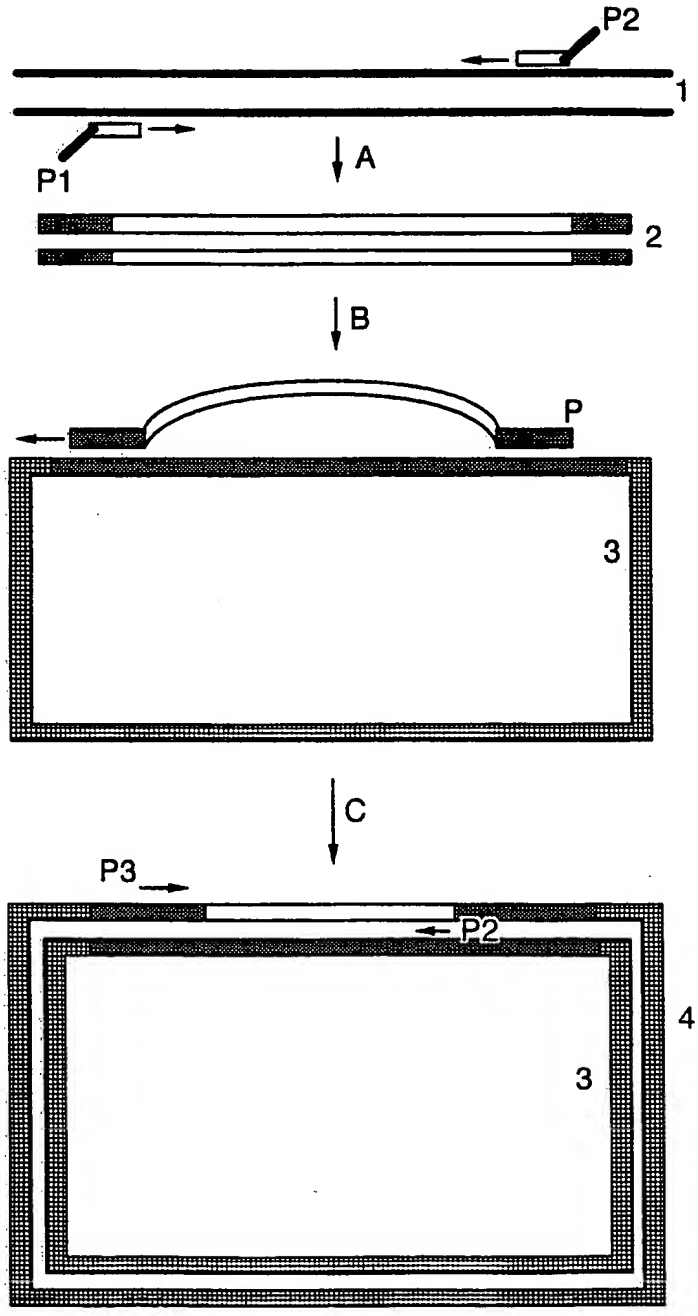
Fig.3.



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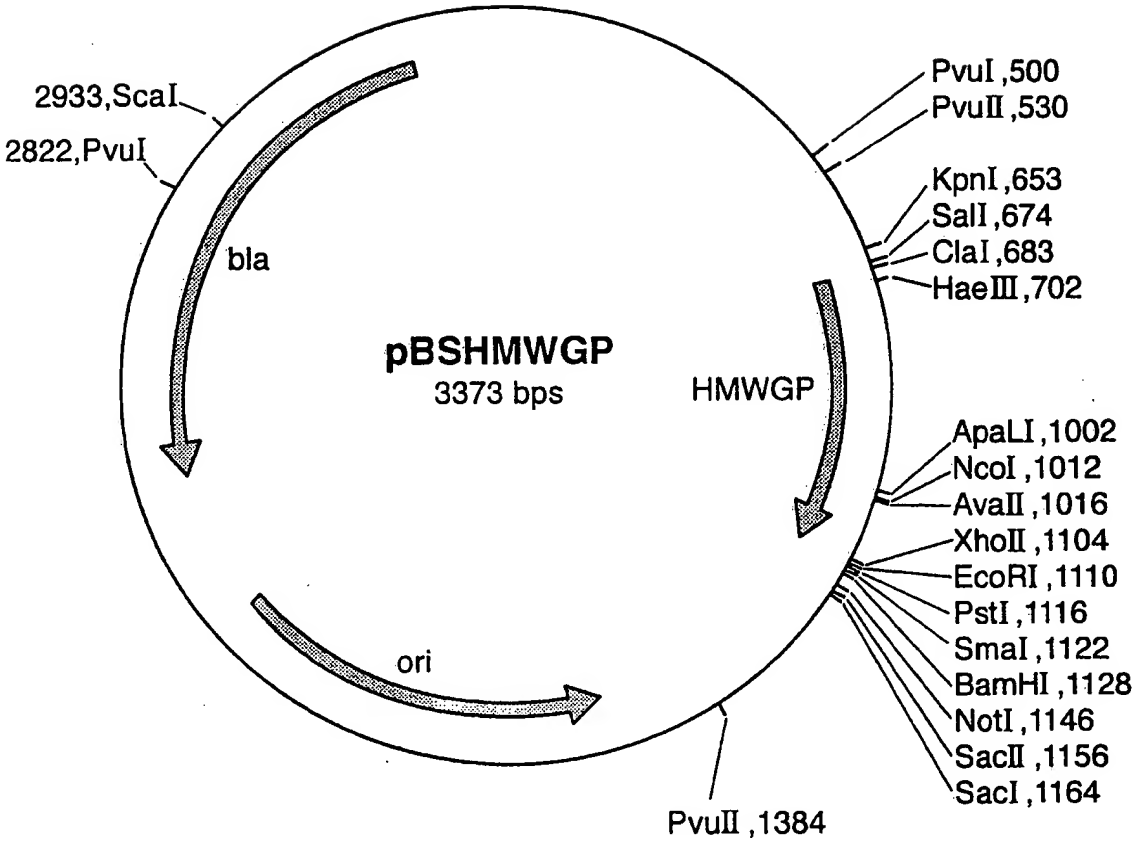
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Fig.5.

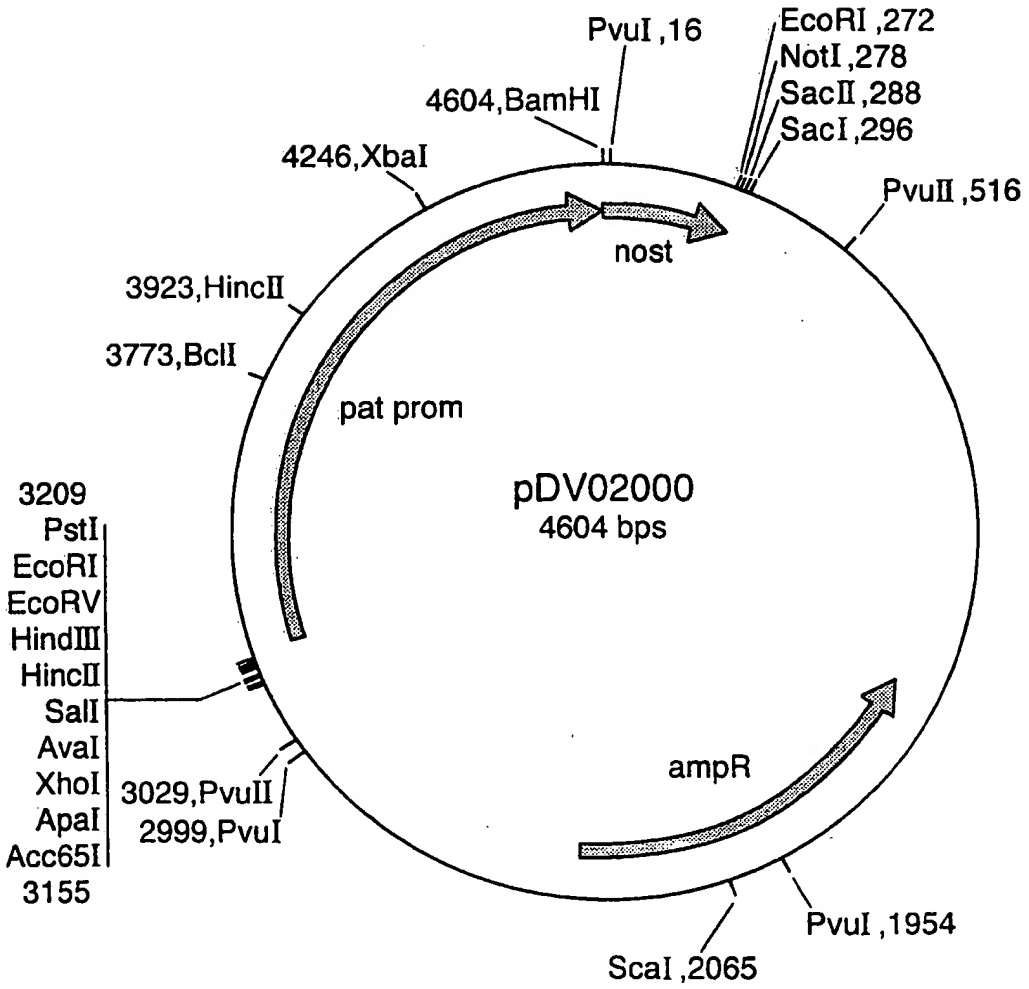
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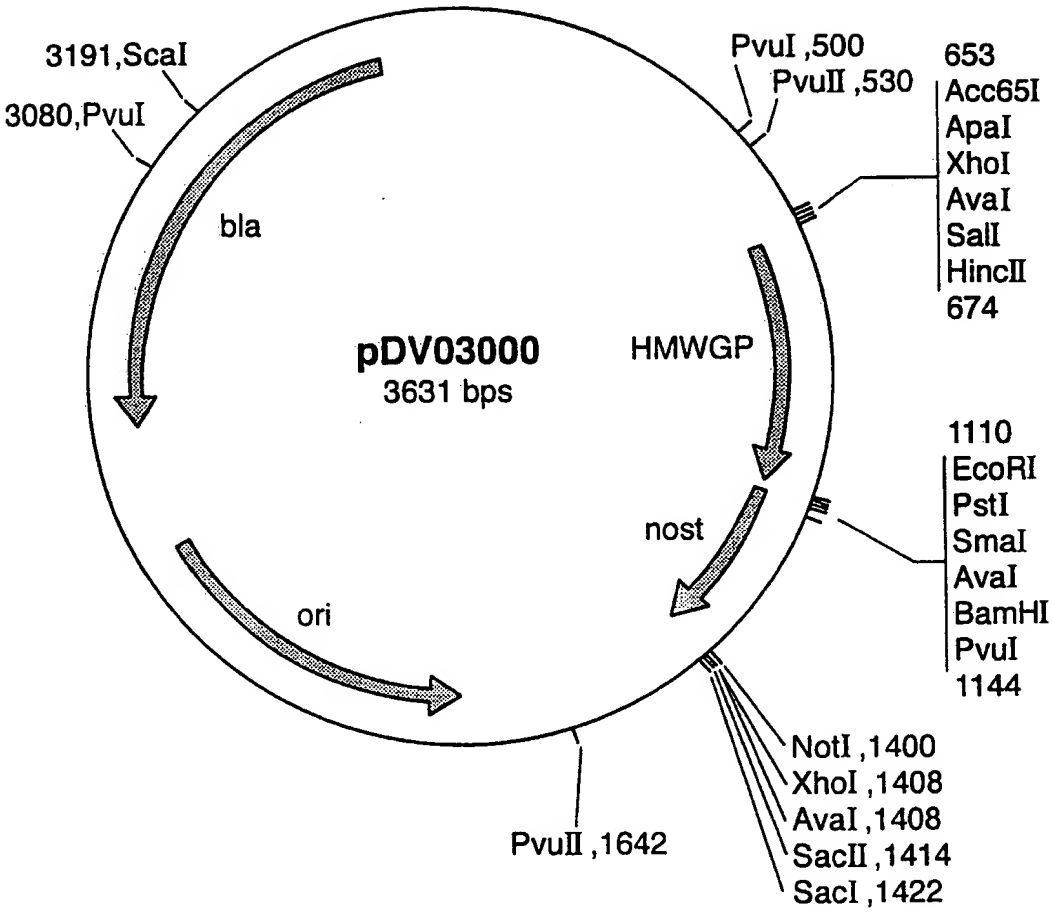
Fig.6.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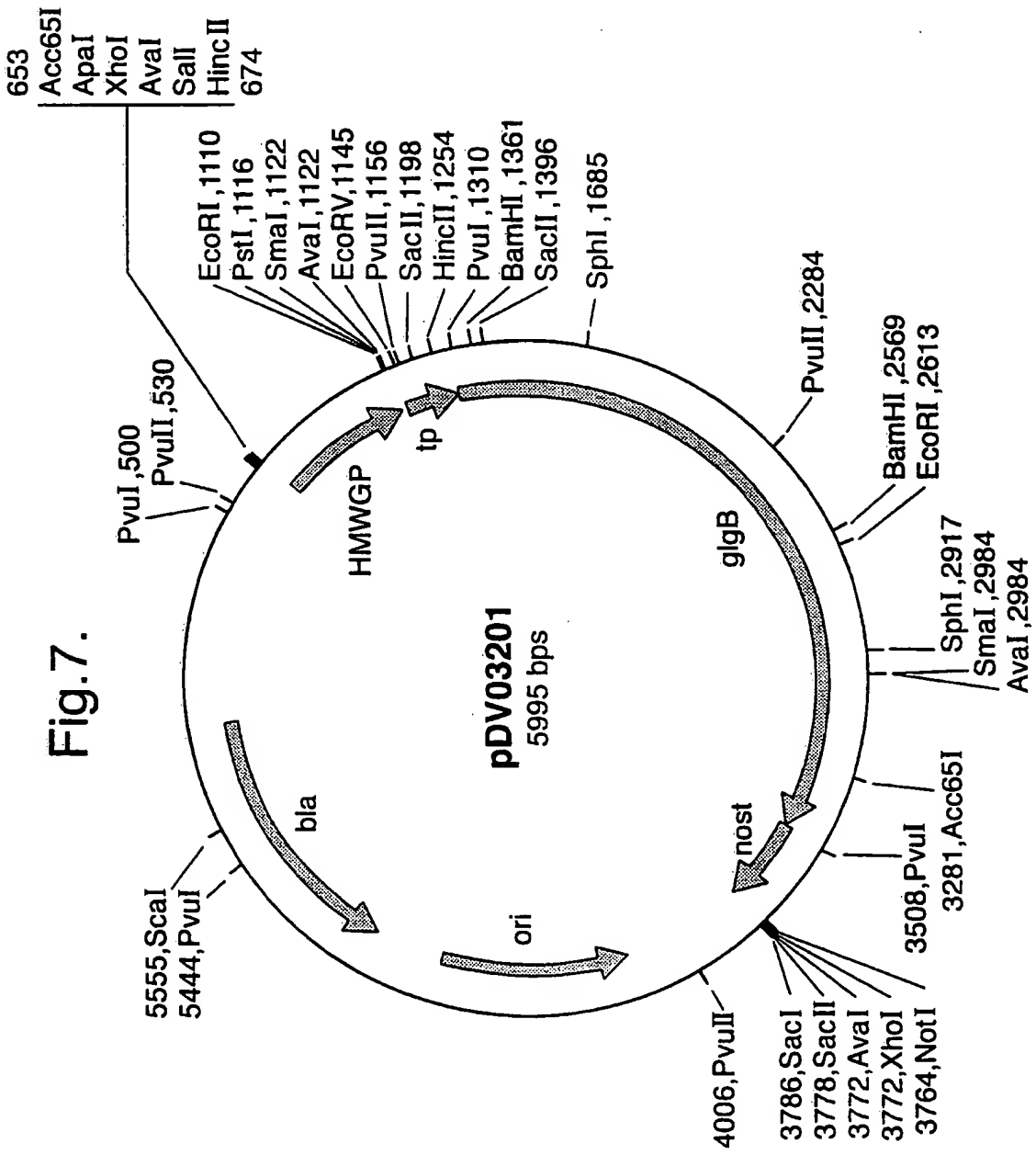
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Fig.7.

Fig.8.

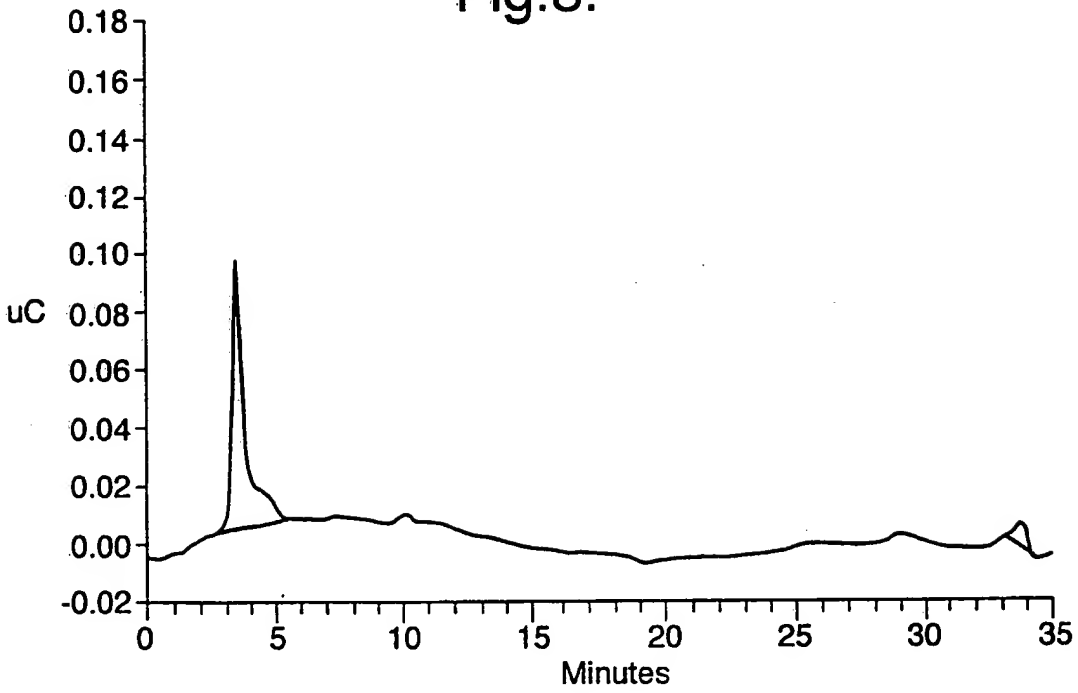
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Fig.9.

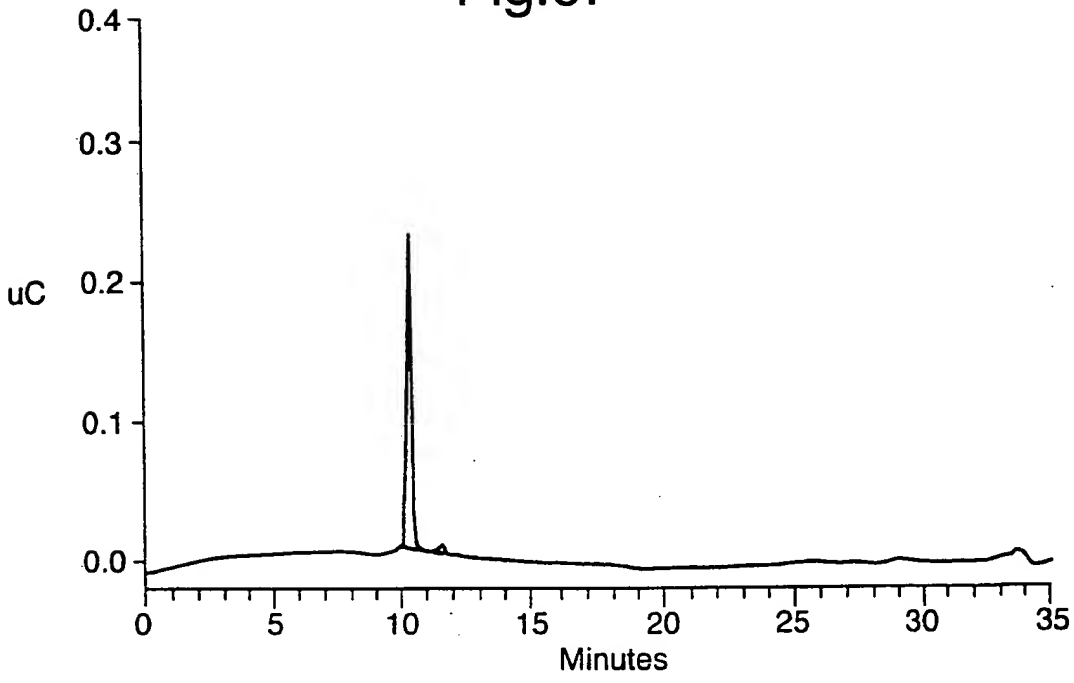
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Fig.10.

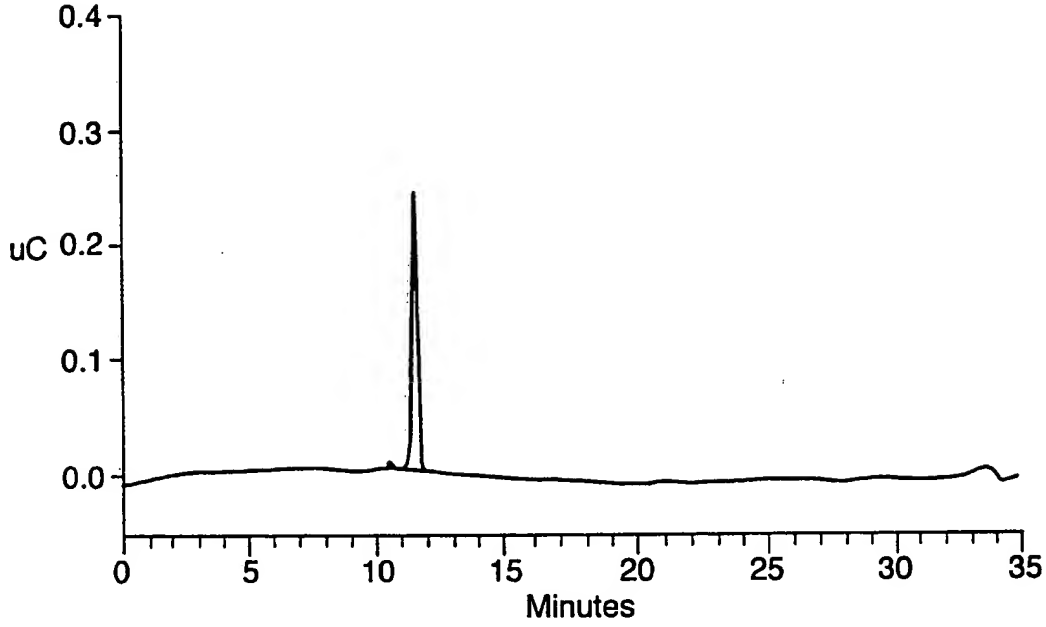
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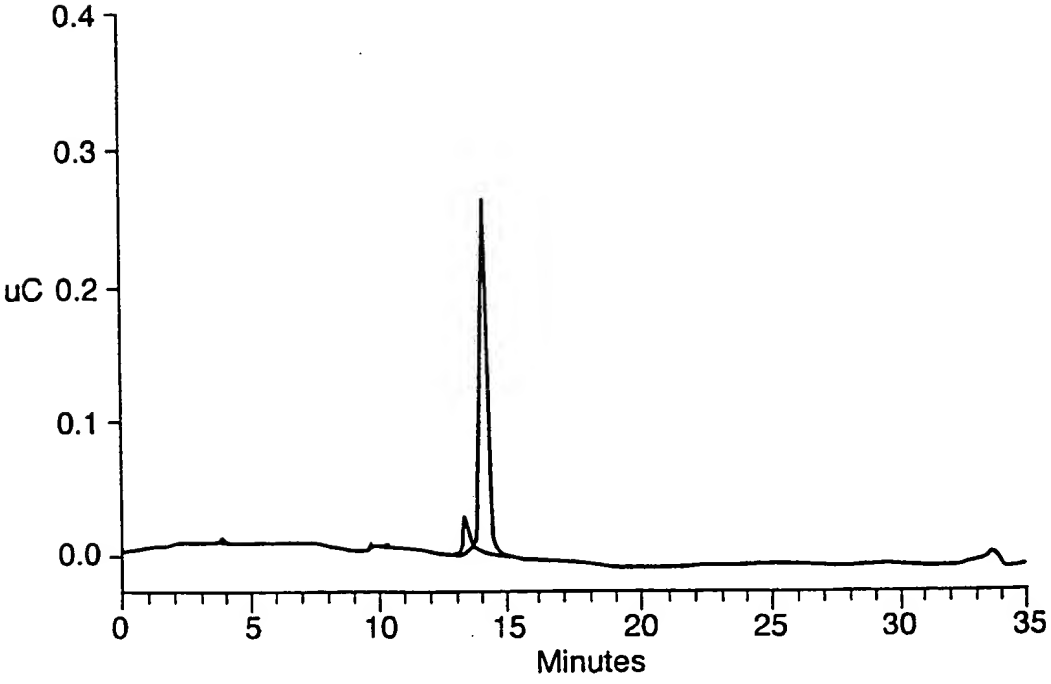
Fig.11.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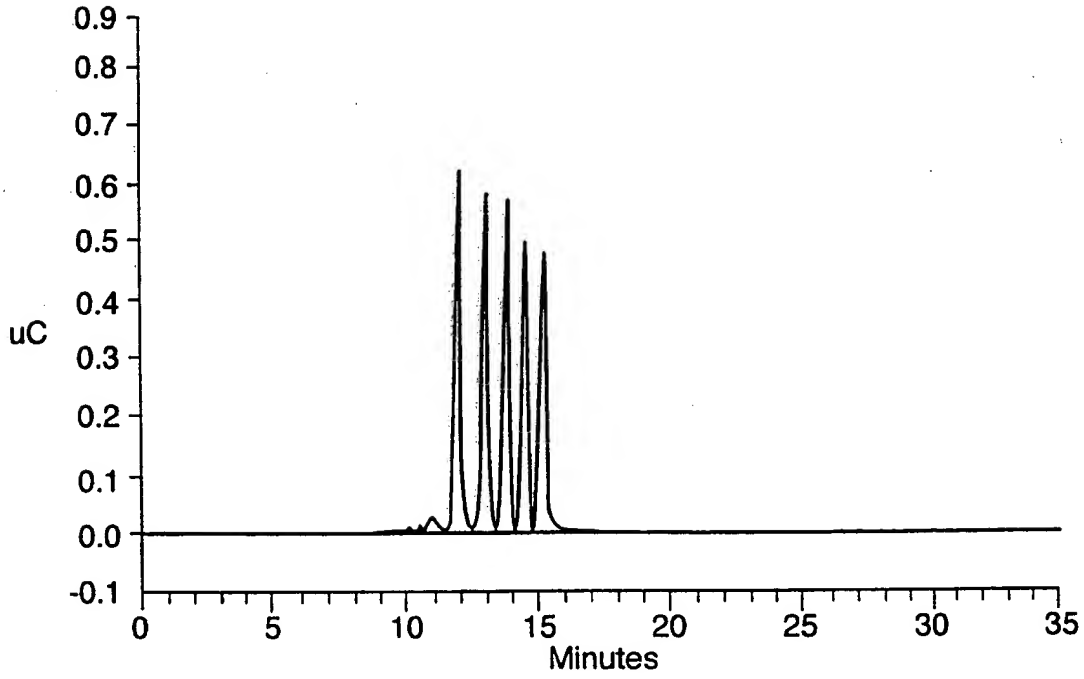
Minutes

Fig.12.



RD-ATC-21

SEQUENCE LISTING

(1) **GENERAL INFORMATION**

(i) **APPLICANTS:**

(A) NAME:	Advanced Technologies (Cambridge) Limited
(B) STREET:	Globe House
(C) CITY:	1 Water Street
(D) STATE:	London
(E) COUNTRY:	England
(F) POSTAL CODE:	WC2R 3LA

(ii) **TITLE OF INVENTION:** Genetically Modified Plants with altered Starch

(iii) **NUMBER OF SEQUENCES:** 9

(iv) **CORRESPONDENCE ADDRESS:**

(A) ADDRESSEE:	British American Tobacco (Investments) Limited
(B) STREET:	Regents Park Road
(C) CITY:	Southampton
(D) STATE:	Hampshire
(E) COUNTRY:	England
(F) POSTAL CODE:	SO15 8TL

(v) **COMPUTER READABLE FORM:**

(A) MEDIUM TYPE:	Diskette 3.50 inch
(B) COMPUTER:	Compaq Deskpro
(C) OPERATING SYSTEM	MS-DOS Windows 95
(D) SOFTWARE:	Microsoft Word 97

(vi) **CURRENT APPLICATION DATA:**

(A) APPLICATION NUMBER:	Not yet known
(B) CLASSIFICATION:	Not yet known

(viii) **ATTORNEY/AGENT INFORMATION:**

(A) NAME:	Mrs. M.R. Walford / Mr.K.J.H. MacLean
(B) REFERENCE:	RD-ATC-21

(ix) **TELECOMMUNICATION INFORMATION:**

(A) TELEPHONE:	01703 777155
(B) TELEFAX:	01703 779856

(2) **INFORMATION FOR SEQ. ID. NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	1467 bps
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** cDNA to mRNA

(vii) **IMMEDIATE SOURCE:**
(B) CLONE: pBS17R

(ix) **FEATURES:**
(A) NAME: CDS ssu transit peptide
(B) LOCATION: 1 to 169

(ix) **FEATURES:**
(A) NAME: CDS *glgC16*
(B) LOCATION: 172 to 146

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:1:

```
   1 ATGGCTTCTA TGATATCCTC TTCAGCTGTG ACTACAGTCA GCCGTGCTTC  50
  51 TACGGTGCAA TCGGCCGCGG TGGCTCCATT CGGCGGCCTC AAATCCATGA 100
 101 CTGGATTCCC AGTTAAGAAG GTCAACACTG ACATTACTTC CATTACAAGC 150
 151 AATGGTGGAA GAGTAAAGTG CATGCTTAGT TTAGAGAAGA ACGATCACTT 200
 201 AATGTTGGCG CGCCAGCTGC CATTGAAATC TGTTGCCCTG ATACTGGCGG 250
 251 GAGGACGTGG TACCCGCCTG AAGGATTTAA CCAATAAGCG AGCAAAACCG 300
 301 GCCGTACACT TCGGCGGTAA GTTCCGCATT ATCGACTTTG CGCTGTCTAA 350
 351 CTGCATCAAC TCCGGGATCC GTCGTATGGG CGTGATCACC CAGTACCAGT 400
 401 CCCACACTCT GGTGCAGCAC ATTCAGCGCG GCTGGTCATT CTTCAATGAA 450
 451 GAAATGAACG AGTTTGTCGA TCTGCTGCCA GCACAGCAGA GAATGAAAGG 500
 501 GGAAAACTGG TATCGCGGCA CCGCAGATGC GGTCACCCAA AACCTCGACA 550
 551 TTATCCGTCG TTATAAAGCG GAATACGTGG TGATCCTGGC GGGCGACCAT 600
 601 ATCTACAAGC AAGACTACTC GCGTATGCTT ATCGATCACG TCGAAAAAGG 650
 651 TGTACGTTGT ACCGTTGTTT GTATGCCAGT ACCGATTGAA GAAGCCTCCG 700
 701 CATTTGGCGT TATGGCGGTT GATGAGAACG ATAAAACTAT CGAATTCGTG 750
 751 GAAAAACCTG CTAACCCGCC GTCAATGCCG AACGATCCGA GCAAATCTCT 700
 801 GGCGAGTATG GGTATCTACG TCTTTGACGC CGACTATCTG TATGAACTGC 750
 851 TGGAAGAAGA CGATCGCGAT GAGAACTCCA GCCACGACTT TGGCAAAGAT 900
```

```
 901 TTGATTCCCA AGATCACCGA AGCCGGTCTG GCCTATGCGC ACCCGTTCCC 950
 951 GCTCTCTTGC GTACAATCCG ACCCGGATGC CGAGCCGTAC TGGCGCGATG 1000
1001 TGGGTACGCT GGAAGCTTAC TGGAAAGCGA ACCTCGATCT GGCCTCTGTG 1050
1051 GTGCCGAAAC TGGATATGTA CGATCGCAAT TGGCCAATTC GCACCTACAA 1100
1101 TGAATCATTA CCGCCAGCGA AATTCGTGCA GGATCGCTCC GGTAGCCACG 1150
1151 GGATGACCCT TAACTCACTG GTTTCCGACG GTTGTGTGAT CTCCGGTTCG 1200
1201 GTGGTGGTGC AGTCCGTTCT GTTCTCGCGC GTTCGCGTGA ATTCATTCTG 1250
1251 CAACATTGAT TCCGCCGTAT TGTTACCGGA AGTATGGGTA GGTCGCTCGT 1300
1301 GCCGTCTGCG CCGCTGCGTC ATCGATCGTG CTTGTGTTAT TCCGGAAGGC 1350
1351 ATGGTGATTG GTGAAAACGC AGAGGAAGAT GCACGTCGTT TCTATCGTTC 1400
1401 AGAAGAAGGC ATCGTGCTGG TAACGCGCGA AATGCTACGG AAGTTAGGGC 1450
1451 ATAAACAGGA GCGATAA                                    1467
```

(2) **INFORMATION FOR SEQ. ID. NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	421 base pairs
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Double stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Genomic DNA

(ix) **FEATURES:**

(A) NAME:	Triticum aestivum
(B) LOCATION:	var CIMMYT

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:2:

```
   1 CCCAGCTTTG AGTGGCCGTA GATTTGCAAA AGCAATGGCT AACAGACACA  50
  51 TATTCTGCCA AACCCCAAGA AGGATAATCA CTTTTCTTAG ATAAAAAAGA 100
 101 ACAGACCAAT ATACAAACAT CCACACTTCT GCAAACAATA CATCAGAACT 150
 151 AGGATTACGC CGATTACGTG GCTTTAGCAG ACTGTCCAAA AATCTGTTTT 200
 201 GCAAAGCTCC AATTGCTCCT TGCTTATCCA GCTTCTTTTG TGTTGGCAAA 250
```

```
251 CTGCGCTTTT CCAACCGATT TTGTTCTTCT CGCGCTTTCT TCTTAGCCTA 300

301 AACAAACCTC ACCGTGCACG CAGCCATGGT CCTGAACCTT CACCTCGTCC 350

351 CTATAAAAGC CTAGCCAACC TTCACAATCT TATCATCACC CACAACACCG 400

401 AGCACCACAA ACTAGAGATC C                                421
```

(2) **INFORMATION FOR SEQ. ID. NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	45 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 3' end of ssu transit peptide
(B) LOCATION:	1 to 19

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 5' end of *glgB* CDS
(B) LOCATION:	20 to 43

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:3:

1 TGGTGGAAGA GTAAAGTGCA TGTCCGATCG TATCGATAGA GACGT

(2) **INFORMATION FOR SEQ. ID. NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	51 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Domain complimentary to 3' end of *glgC* coding sequence
(B) LOCATION:	1 to 19

(ix) **FEATURES:**
(A) NAME: Domain complimentary to 3' end of *glgB* CDS
(B) LOCATION: 26 to 51

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID NO:4:

1 TCGCTCCTGT TTATGCCCTA GATCTTCATT CTGCCTCCCG AACCAGCCAG 50

51 A 51

(2) **INFORMATION FOR SEQ. ID. NO:5:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 33 bases
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single stranded
(D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Complimentary to 5' end of ssu transit peptide
(B) LOCATION: 11 to 33

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:5:

1 ACGTAGATCT ATGGCTTCTA TGATATCCTC TTC 33

(2) **INFORMATION FOR SEQ. ID. NO:6:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 36 bases
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single stranded
(D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Homologous to 5' end of High Molecular Weight Glutenin Promoter
(B) LOCATION: 10 to 36

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:6:

1 GACATCGATC CCAGCTTTGA GTGGCCGTAG ATTTGC 36

(2) **INFORMATION FOR SEQ. ID. NO:7:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	39 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Complimentary to 3' end of High Molecular Weight Glutenin Promoter
(B) LOCATION:	10 to 39 Promoter

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:7:

1 GACGAATTCG GATCTCTAGT TTGTGGTGCT CGGTGTTGT 39

(2) **INFORMATION FOR SEQ. ID. NO:8:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	32 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**

(A) NAME:	Homologous to 5' end of Nopaline synthase terminator
(B) LOCATION:	9 to 32

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:8:

1 CAGGATCCGA ATTTCACCCG ATCGTTCAAA CA 32

(2) **INFORMATION FOR SEQ. ID. NO:9:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) LENGTH:	50 bases
(B) TYPE:	Nucleotide
(C) STRANDEDNESS:	Single stranded
(D) TOPOLOGY:	Linear

(ii) **MOLECULE TYPE:** Oligonucleotide primer

(ix) **FEATURES:**
(A) NAME: Complimentary to 3' end of nopaline synthase terminator
(B) LOCATION: 23 to 50

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:9:

1 GACCGCGGC TCGAGGCGGC CGCCCGATCT AGTAACATAG ATGACACCGC 50

INTERNATIONAL SEARCH REPORT

Internation pplication No
PCT/GB 99/03762

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/54 A01H5/00 C08B30/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C08B A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KORTSTEE A J ET AL: "Expression of Escherichia coli branching enzyme in tubers of amylose-free transgenic potato leads to an increased branching degree of the amylopectin." PLANT JOURNAL, (1996 JUL) 10 (1) 83-90. , XP002135212 the whole document	1-22,24
X	WO 98 44780 A (EXSEED GENETICS, LLC, USA) 15 October 1998 (1998-10-15) see the whole document; esp. pp.23-25, fig. 26,27	1-22,24

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 April 2000	27/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB 99/03762

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KORTSTEE A J ET AL: "The influence of an increased degree of branching on the physicochemical properties of starch from genetically modified potato" CARBOHYDRATE POLYMERS, (OCT 1998) VOL. 37, NO. 2, PP. 173-184. PUBLISHER: ELSEVIER SCI LTD, THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD OX5 1GB, OXON, ENGLAND. ISSN: 0144-8617., XP004141125 the whole document	15-19
A	WO 92 11382 A (CALGENE INC) 9 July 1992 (1992-07-09) cited in the application the whole document	1-24
A	WO 94 09144 A (ZENECA LTD) 28 April 1994 (1994-04-28) cited in the application see the whole document; esp. p.33/34, examples, claims	1-24
A	WO 94 11520 A (ZENECA LTD ;KEELING PETER LEWIS (GB)) 26 May 1994 (1994-05-26) cited in the application see the whole document; esp. pp.18-26	1-24
A	SHEWMAKER C K ET AL: "EXPRESSION OF ESCHERICHIA COLI GLYCOGEN SYNTHASE IN THE TUBERS OF TRANSGENIC POTATOES (SOLANUM TUBEROSUM) RESULTS IN A HIGHLY BRANCHED STARCH" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 104, 1 January 1994 (1994-01-01), pages 1159-1166, XP002033871 ISSN: 0032-0889 cited in the application the whole document	1-24
A	WO 97 22703 A (DU PONT ;HUBBARD NATALIE LOUISE (US); KLEIN THEODORE MITCHELL (US)) 26 June 1997 (1997-06-26) the whole document	1-24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB 99/03762

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